Diosgenin induces apoptosis in HeLa cells via activation of caspase pathway

Rui HUO[1,2], Qiu-li ZHOU[2], Ben-xiang WANG[3], Shin-ichi TASHIRO[4], Satoshi ONODERA[4], Takashi IKEJIMA[1,5]

[1]*China-Japan Research Institute of Medical and Pharmaceutical Sciences, Shenyang Pharmaceutical University, Shenyang 110016, China;* [2]*Jilin University, Changchun 130021;* [3]*Changchun College of Traditional Chinese Medicine, Changchun 130021, China;* [4]*Department of Clinical and Biomedical Sciences, Showa Pharmaceutical University, Tokyo 194-8543, Japan*

ABSTRACT

AIM: To investigate the mechanism of diosgenin-induced HeLa cell apoptosis. **METHODS:** HeLa cell growth was measured by MTT method. Apoptosis was detected by electron microscopy and agarose gel electrophoresis. Ratio of apoptotic cells was measured by APO-BRDU kit. Cell cycle distribution and changes of mitochondrial membrane potential were monitored by flow cytometry. Caspase activities were assayed by caspase apoptosis detection kit. Western blot analysis was used to evaluate the level of mitochondrial Bcl-2 expression. **RESULTS:** Diosgenin inhibited HeLa cell growth. HeLa cells treated with diosgenin showed typical characteristics of apoptosis including the morphological changes and DNA fragmentation. Caspase family inhibitor (z-VAD-fmk), caspase-9 inhibitor (Ac-AAVALPAVLLALLAPLEHD-CHO), and caspase-3 inhibitor (z-DEVD-fmk) partially prevented diosgenin-induced apoptosis, but not caspase-8 inhibitor (z-IETD-fmk) and caspase-10 inhibitor (z-AEVD-fmk). Diosgenin caused reduction of mitochondrial membrane potential and down-regulated Bcl-2 expression. **CONCLUSION:** Diosgenin induced HeLa cell apoptosis through caspase pathway.

INTRODUCTION

Diosgenin, a plant steroid (5α-spirosten-3β-ol) was first isolated from *Dioscorea tokoro* in 1930s[1]. It has been reported that diosgenin induces differentiation of human erythroleukemia cell line (HEL TIB 180) through changing lipoxygenase activities[2,3]. In addition, diosgenin was used to treat osteoporosis in the ovariectomized adult rat model[4]. Recently, diosgenin has been reported to induce apoptosis and cell cycle arrest in human osterosarcoma 1547 cell line[5]. However, the precise mechanism of diosgenin-induced apoptosis is still unclear.

The caspase family of aspartate-specific cysteine proteases have been demonstrated to be important mediators in apoptotic pathway[6]. Caspases, a family of at least 14 cysteine proteases, are synthesized as proenzymes, which are proteolytically cleaved into active heterodimers. Caspases can be grouped according to their substrate specificities, which are mainly defined by the amino acids preceding the cleavage site of aspartic acid residue[7,8].

Mitochondria plays a key role in apoptotic process[9]. In apoptosis, there is an alteration of mitochondrial membrane permeability, which causes the loss of mitochondrial membrane potential and translocation of the cytochrome c into cytoplasm. The cytochrome c in turn activates caspase cascade. Bcl-2 is a membrane

protein located mainly at the outer membrane of mitochondria and it appears antiapoptotic function. Many reports have demonstrated that one possible role of Bcl-2 in prevention apoptosis is to block cytochrome c release from mitochondria[10-13]

In the present study, we investigated the participation of caspases, the changes of mitochondrial membrane potential and Bcl-2 expression in HeLa cell apoptosis induced by diosgenin.

MATERIALS AND METHODS

Chemical reagents Diosgenin purchased from Sigma (MO, USA) was dissolved in ethanol and Me_2SO (3:1), the final concentration of which, less than 0.1 %, did not cause morphological changes and cytotoxicity.

Cell culture HeLa cells were obtained from American Type Culture Collection (ATCC, #CRL-1872) (MD, USA) and were cultured in RPMI-1640 medium (GIBCO, NY, USA) supplemented with 10 % heat inactivated (56 ℃, 30 min) fetal calf serum (Beijing Yuanheng Shengma Research Institution of Biotechnology, Beijing, China), *L*-glutamin 2 mol/L (GIBCO), benzylpenicillin 100 kU/L, and streptomycin 100 mg/L (GIBCO) at 37 ℃ in 5 % CO_2 .

Cell growth inhibition test HeLa cells (1.5×10^8 cells/L) were seeded in the 96-well plates (NUNK™, Denmark). After 24 h, the cells were treated with various concentrations of diosgenin (15-120 µmol/L) for 12, 24, and 36 h, respectively. Thiazolyl blue (MTT) (Sigma, MO, USA) test[14] was performed to detect the inhibition of cell growth using an enzyme-linked immunosorbent assay plate reader (TECAN, Austria). HeLa cells were preincubated with or without various concentrations of caspase inhibitors, caspase family inhibitor (z-VAD-fmk), caspase-8 inhibitor (z-IETD-fmk) (Enzyme System, USA), caspase-10 inhibitor (z-AEVD-fmk) (Techne, USA), caspase-9 inhibitor (Ac-AAVAL-PAVLLALLAPLEHD-CHO) (Santa Cruz Biote-chnology, USA), or caspase-3 inhibitor (z-DEVD-fmk) (Calbiochem, USA) for 2 h. Then, the cells were treated with additional diosgenin 30 µmol/L for 36 h.

Observation of morphological changes HeLa cells were seeded in culture-plates. After 24 h, the cells were treated with diosgenin 30 µmol/L for 0, 12, 24, and 36 h, respectively. The adherent and floating cells were collected. The cells were fixed, dehydrated in ethanol and embedded in epoxy resin (Epon 812). Morphological changes were observed by electron micros-copy (JEM-1200EX, Japan).

Determination of DNA fragmentation DNA extraction and electrophoresis were performed as described previously[15]. In brief, HeLa cells containing adherent and floating ones were collected by centrifugation at $1000 \times g$ for 5 min. The cell pellet was suspended in cell lysis buffer (Tris-HCl 10 mmol/L pH 7.4, edetic acid 10 mmol/L pH 8.0, Triton-X 100 0.5 %) and kept at 4 ℃ for 10 min. The lysate was centrifuged at $25\,000 \times g$ for 20 min. The supernatant was incubated with RNase A 40 µg/L (Sigma) at 37 ℃ for 1 h, then incubated with proteinase K 40 µg/L (Merck, USA) at 37 ℃ for 1 h. The solution was mixed with NaCl 0.5 mol/L and 50 % 2-propanol overnight at -20 ℃, then centrifuged at $25\,000 \times g$ for 15 min. After drying, DNA was dissolved in TE buffer (Tris-HCl 10 mmol/L pH 7.4, edetic acid 1 mmol/L pH 8.0) and separated by 2 % agarose gel electrophoresis at 100 V for 50 min.

Quantification of apoptotic cells Ratio of apoptotic cells was measured by flow cytometry (APO-BRDU kit, Becton Dickinson, USA) as described by manufacturer's instructions. In brief, HeLa cells treated with diosgenin 30 µmol/L for 0, 12, 24, and 36 h were collected and resuspended in 5 mL of PBS with 1 % paraformaldehyde and placed on ice for 15 min. The cells were washed with PBS and fixed in 70 % ethanol on ice for 30 min, then washed with PBS again. The cells were incubated with DNA labeling solution 50 µL at 37 ℃ for 60 min, then washed with PBS. The cells were incubated with antibody solution 0.1 mL in dark for 30 min at room temperature.

Flow cytometric analysis of cell cycle distribution Cell cycle distribution was detected using CycleTEST PLUS DNA Reagent kit (Becton Dickinson, USA). In brief, HeLa cells (1×10^6) were collected and fixed in 70 % ethanol at -20 ℃ for 18 h after treatment with diosgenin 30 µmol/L for 0, 12, 24, and 36 h. The cells were washed with PBS two times, then pelleted by centrifugation at $400 \times g$. The cell pellets were incubated with solution A 250 µL (trypsin in a spermine tetrahydrochloride detergent buffer) at room temperature for 10 min, solution B 200 µL (trypsin inhibitor and ribonuclease A in citrate stabilizing buffer with spermine tetrahydrochloride) at room temperature for 10 min and solution C 200 µL (propidium iodide and spermine tetrahydrochloride in cirtrate stabilizing buffer) in the dark condition at 4 ℃ for 10 min.

Assay of caspase activities HeLa cells were treated with 30 µmol/L diosgenin for 0, 12, 24, and 36

h, then caspase-3 and caspase-8 activities were measured by Caspase Apoptotosis Detection Kit (Santa Cruz Biotechnology). In brief, cells (1×10^6) were pelleted by centrifugation , washed with PBS two times and incubated in 500 μL lysis buffer on ice for 10 min, then 1×reaction buffer and 10 μL DEVD-AFC or IEVD-AFC substrates was added to lysis buffer. The reaction mixtures were incubated at 37 °C for 1 h.

Flow cytometric analysis of mitochondrial membrane potential changes[16] HeLa cells were treated with diosgenin 30 μmol/L for 0, 12, 24, and 36 h. The cells were centrifuged, resuspended in PBS containing 0.5 % FCS, and incubated with 40 nmol/L 3,3-dihexyloxacarbocyanine iodide [$DiOC_6$(3), Eugene, OR, USA] at 37 °C for 15 min, then changes of mitochondrial potential were detected by flow cytometry.

Western blot analysis of Bcl-2 expression HeLa cells were treated with 30 μmol/L diosgenin for 0, 12, 24, 36 h. Both adherent and floating cells were collected and frozen at -80 °C. Western blot analysis were performed as previously described[17] with some modification. Briefly, the cell pellets were resuspended in lysis buffer, including Hepes 50 mmol/L pH 7.4, Triton-X 100 1 %, sodium orthovanada 2 mmol/L, sodium fluoride 100 mmol/L, edetic acid 1 mmol/L, egtazic acid 1 mmol/L, PMSF 1 mol/L, aprotinin (Sigma) 0.1 g/L, leupeptin (Sigma) 0.01 g/L, then lysed in 4 °C for 1 h. After 13 000×*g* centrifugation for 10 min, the protein content of supernatant was determined using Bio-Rad protein assay reagent (Bio-Rad, USA). The protein lysates were separated by electrophoresis in 12 % SDS polyacrylamide gel and blotted onto nitrocellulose membrane. Protein expression was detected by rabbit primary polyclonal anti Bcl-2 antibody (Oncogene, USA) and secondary polyclonal antibody conjugated with peroxidase (goat anti-rabbit IgG) (Santa Cruz Biotechnology).

Statistics Data were expressed as mean±SD and analyzed by *t*-test.

RESULTS

Inhibitory effect of diosgenin on cell growth Diosgenin, ranging from 15-120 μmol/L, inhibited growth of HeLa cells. Diosgenin 30 μmol/L inhibited HeLa cell growth in a time-dependent manner, however, diosgenin 15, 60, and 120 μmol/L did not show such ability (Fig 1). By light microscopy, we observed a decrease in the total number of cells and an accumulation of cells
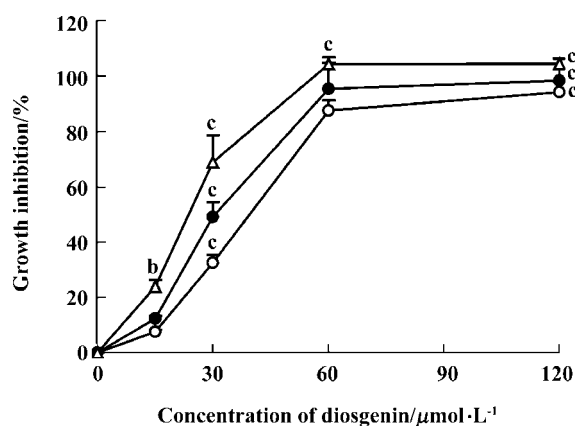

Fig 1. Inhibitory effect of diosgenin on HeLa cell growth. The cells (1.5×10^8 cells/L) were incubated with various concentrations of diosgenin for 12 h (○), 24 h (●), 36 h (△). *n*=4. Mean±SD. $^bP<0.05$, $^cP<0.01$ *vs* control (0 h).

floating in the culture medium, indicating diosgenin-induced cell death. Diosgenin 30 μmol/L inhibited a half maximal growth inhibition, thus we chose 30 μmol/L for the following experiments.

Diosgenin-induced morphological changes and DNA fragmentation To determine whether diosgenin induced HeLa cell death by apoptosis, we examined morphological changes and DNA fragmentation. When HeLa cells were treated with diosgenin 30 μmol/L for 0, 12, 24, and 36 h, morphological changes were observed by electron microscopy. In the control group, the cells exhibited an intact morphology of nucleus and cytoplasm, in contrast, diosgenin-treated cells showed typical characteristics of apoptosis with loss of membrane microvilli, cytoplasmic hypervacuolization, and nuclear fragmentation. HeLa cells incubated with diosgenin 30 μmol/L for 36 h showed a obvious apoptotic pattern of DNA ladder in agarose gel electrophoresis (Fig 2, lane c).

Measurement of diosgenin-induced apoptotic cells In order to further verify cell apoptosis after 30 μmol/L diosgenin treatment, we measured apoptotic cells by APO-BRDU kit. The results indicated that ratios of apoptotic cells were 26.65 % at 12 h, 46.43 % at 24 h, and 65.49 % at 36 h, demonstrating that diosgenin 30 μmol/L induced HeLa cell apoptosis, but not necrosis (Fig 3).

Effect of diosgenin on cell cycle distribution Cell cycle distribution was not changed, but an accumulation of sub-G_0/G_1 phase, associated with apoptosis, was observed after HeLa cells were treated with diosgenin 30 μmol/L for 0, 12, 24, and 36 h (Tab 1).
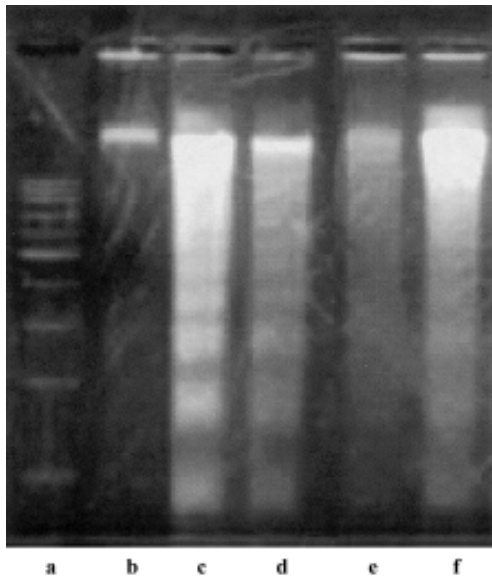
Fig 2. DNA fragmentation induced by diosgenin and blockade of diosgenin-induced DNA fragmentation by caspase inhibitors in HeLa cells. a: Hae-III-digested ΦX-174 DNA fragments were used as molecular marker. b: HeLa cells were incubated with diosgenin 0 μmol/L for 36 h (b). c-f: HeLa cells were incubated with diosgenin 30 μmol/L alone for 36 h (c) or pretreated with Ac-AAVALPAVLLALLAPLEHD-CHO 2.5 μmol/L (d), z-DEVD-fmk 10 μmol/L (e), or z-VAD-fmk 20 μmol/L (f) for 2 h before diosgenin administration.
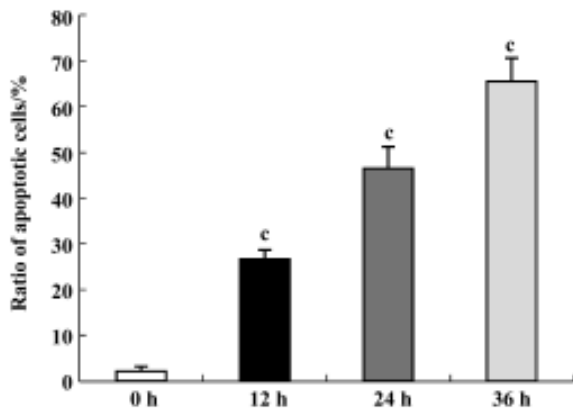

Fig 3. Detection of apoptotic cells by flow cytometry. HeLa cells were treated with diosgenin 30 μmol/L for 0, 12, 24, and 36 h. *n*=3. Mean±SD. $^cP<0.01$ *vs* 0 h.

Effect of caspase inhibitors on diosgenin-induced apoptosis Diosgenin-induced HeLa cell apoptosis was partially inhibited by caspase inhibitors, z-VAD-fmk, z-DEVD-fmk, and Ac-AAVALPAVLLALLAPLEHD-CHO, but not by z-IETD-fmk and z-VEAD-fmk. Inhibitory effect of z-VAD-fmk, Ac-AAVALPAVLLAL-LAPLEHD-CHO, and z-DEVD-fmk was 24.57 %, 21.36 %, and 30.79 %, respectively (Fig 4). In order

Tab 1. Flow cytometric analysis of cell cycle distribution in HeLa cells which were treated with diosgenin 30 μmol/L for 0, 12, 24, and 36 h. *n*=3. Mean±SD. $^cP<0.01$ *vs* 0 h.

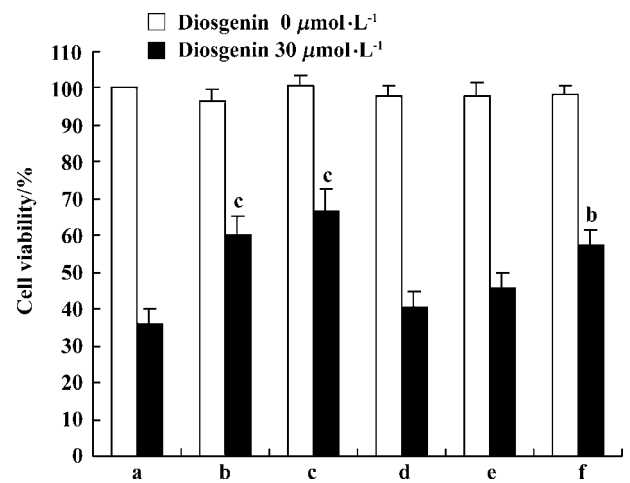| Time/h	Cell cycle distribution			
	Sub G_0/G_1	G_0/G_1	S	G_2/M (%)
0	3.5±1.1	66.3±4.5	21.5±3.4	12.3±2.6
12	19.1±4.2^c	63.1±3.2	25.8±3.4	11.1±3.6
24	36.0±3.8^c	68.5±5.3	19.2±0.9	12.3±4.4
36	51.7±3.0^c	68.0±3.8	20.6±1.9	11.3±1.9

Fig 4. Effect of caspase inhibitors on diosgenin-induced HeLa cell apoptosis. HeLa cells were preincubated with or without different caspase inhibitors for 2 h, then treated with diosgenin 30 μmol/L for 36 h. a: control; b: z-VAD-fmk (20 μmol/L); c: z-DEVD-fmk (10 μmol/L); d: z-IETD-fmk (10 μmol/L); e: z-AEVD-fmk (10 μmol/L); f: Ac-AAVAL-PAVLLALLAPLEHD-CHO (2.5 μmol/L). *n*=3. Mean±SD. $^bP<0.05$, $^cP<0.01$ *vs* diosgenin 0 μmol/L.

to further examine the participation of caspases in diosgenin-induced apoptosis, caspase-3, -8 activities and the effect of caspase inhibitors on DNA fragmentation were assayed. Caspase-3 activity was obviously induced after the cells were treated with diosgenin 30 μmol/L for 36 h, but caspase-8 activity did not change at 12, 24, and 36 h (Tab 2). We also found that z-VAD-fmk, Ac-AAVALPAVLLALLAPLEHD-CHO, and z-DEVD-fmk decreased diosgenin-induced DNA fragmentation (Fig 2d-2f).

Changes of mitochondrial membrane potential in HeLa cells HeLa cells treated with diosgenin 30 μmol/L for 24 and 36 h showed a significant reduction of fluorescence intensity (Fig 5), which reflected the

Tab 2. Activities of caspase-3 and caspase-8 in HeLa cells treated with diosgenin 30 µmol/L for 0, 12, 24, and 36 h. Activities of caspase-3 and caspase-8 were assayed by measuring absorbance at 505 nm upon excitation at 400 nm. *n*=3. Mean±SD. ^cP<0.01 compared with 0 h.

Time/h	$A_{505\ nm}$	
	Caspase-3	Caspase-8
0	2.25±0.50	2.46±0.27
12	2.77±1.05	2.54±0.13
24	3.18±0.58	2.86±0.11
36	5.41±0.59[c]	2.82±0.32

Fig 6. The expression of Bcl-2 in diosgenin-treated Hela cells. HeLa cells were treated with diosgenin 30 µmol/L for 0, 12, 24, and 36 h. The expression of β-actin was internal control.

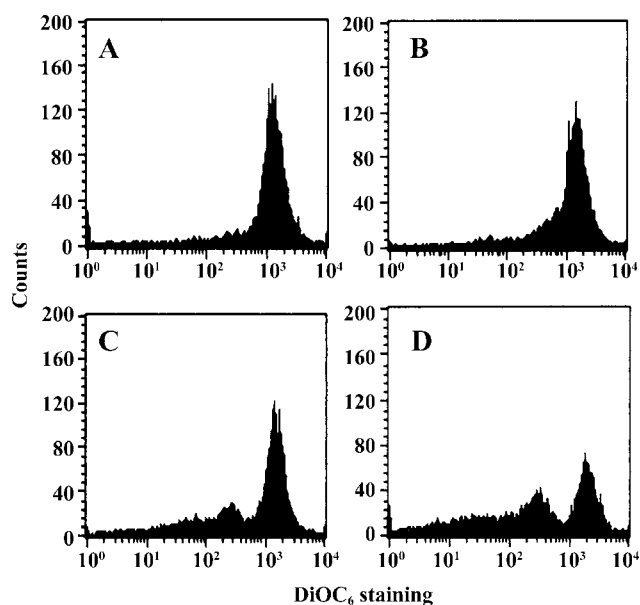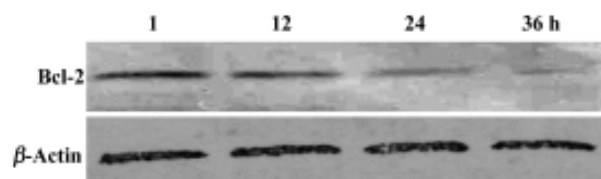**Fig 5. Effect of diosgenin on mitochondrial membrane potential in HeLa cells. HeLa cells were treated with diosgenin 30 µmol/L for 0 h (A), 12 h (B), 24 h (C), 36 h (D), then stained with DiOC₆(3). Changes of mitochondrial membrane potential were detected by flow cytometry.**

collapse of mitochondrial membrane potential.

Effect of diosgenin on Bcl-2 expression HeLa cells were treated with diosgenin 30 µmol/L for 0, 12, 24, and 36 h. After 24 h, Bcl-2 expression began to decrease and was almost undetectable after 36 h (Fig 6).

DISCUSSION

The present study showed that diosgenin induced HeLa cell apoptosis at 30 µmol/L, but not necrosis.

Since caspase plays major roles in apoptotic process, involvement of caspase cascade was examined in diosgenin-induced HeLa cell apoptosis. In our study, caspase family inhibitor (z-VAD-fmk) partially blocked diosgenin-induced HeLa cell apoptosis, indicating that caspase participated in this death process. There are at least two major pathways, initiated by caspase-8 and caspase-9, which initiate caspase cascades in the cells. Death receptors such as Fas induce caspase-8 activation via the adapter molecule, Fas-associated death domain protein. Chemotherapeutic agents and UV irradiation cause release of mitochondrial cytochrome c, which binds to the adapter molecule, apoptotic proteinase activating factor-1 (APAF-1), and this complex along with adenine nucleotides promotes caspase-9 autoactivation. Once activated, these caspases in turn activate executioner caspases, caspase-3, -6, and -7. The active executioners promote apoptosis by cleaving cellular substrates that induce the morphological and biochemical features of apoptosis[18]. Caspase-10 is also an initiator caspase, which shares homologous death-effector domains with caspase-8, but caspase-10 and caspase-8 may have different apoptosis substrates, therefore have potentially distinct roles in apoptosis[19]. We examined the roles of caspase-3, -8, -9, -10 in diosgenin-induced HeLa cell apoptosis. Based on the results of caspase activities and its inhibitors' anti-apoptotic effects on diosgenin-induced cell apoptosis, we concluded that caspase-9 and caspase-3 played key roles in the post-mitochondrial apoptotic pathway. On the other hand, diosgenin passes through cell membrane because of its conformation (5α-spirosten-3β-ol), therefore it is possible that diosgenin induces apoptosis via activation of caspase-9, but not caspase-8 which exisits in the vicinity of cell membrane.

One of the major pathways for caspase activation involves the participation of mitochondria. Bcl-2, a mitochondrial protein, inhibits apoptotic process and promotes cell survival[20,21]. Since diosgenin decreased mitochodrial membrane potential and Bcl-2 expression

and activated caspase-9 cascade, further studies on cytochrome c release remains to be conducted.

Dioscin, a derivative compound from diosgenin, has been reported to induce HeLa cell apoptosis through activating caspase-9 and caspase-3[22]. The mechanism of dioscin-induced apoptosis is similar to that of diosgenin. Dioscin induces HeLa cell apoptosis at lower doses attributed to the subtle difference of molecular conformation between diosgenin and dioscin.

Diosgenin-induced apoptotic signal goes through caspase pathways in HeLa cells, however, it is possible that other apoptotic pathways may also participate in this process.

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